Biochem. J. (1967) **105**, 927
Printed in Great Britain

Assimilation of Glucose Carbon in Subcellular Rat Brain Particles *in vivo* and the Problem of Axoplasmic Flow

By R. VRBA*
Medical Research Council Neuropsychiatric Research Unit, Carshalton, Surrey

(*Received* 3 *March* 1967)

1. Rats were injected with [U-^{14}C]glucose and the content of ^{14}C in proteins and lipids of the cerebral P_1 ('nuclear'), P_2 ('mitochondrial'), P_3 ('microsomal') and high-speed supernatant fractions was measured 7, 22 and 93hr. after injection of labelled glucose. 2. The crude brain mitochondrial fractions (P_2) were subfractionated on continuous sucrose gradients (0·32–1·8M-sucrose) and the ^{14}C content of the proteins and lipids of about 20 subfractions was measured. 3. About 40–50% of the ^{14}C assimilated by brain proteins was found in the P_2 ('mitochondrial') fraction. About 68–70% of the ^{14}C assimilated by brain lipids was also recovered from the lipids of the P_2 fraction. 4. Between 22 and 93hr. after injection of [U-^{14}C]glucose both the amount of ^{14}C in the protein of the P_2 ('mitochondrial') fraction and the specific activity of this protein increased. The specific activity of the protein of all other particulate fractions (P_1, P_2 and P_3) and subfractions (obtained from sucrose-density-gradient subfractionation of fraction P_2) when related to the specific activity of the high-speed supernatant protein also increased during 93hr. after injection of [U-^{14}C]glucose. The amount of ^{14}C in the protein of the high-speed supernatant and the specific activity of this protein decreased during the same period. 5. The distribution of ^{14}C in the lipids of all subcellular particulate fractions remained unchanged during the period 22–93hr. after injection of [U-^{14}C]glucose. 6. It was concluded that a diffusion occurs of some supernatant proteins into subcellular particulate matter of the cerebrum and no significant preference for any subcellular particulate matter was observed. The lipids occur in the cerebrum mainly in a non-diffusible state, which is consistent with the view that they form almost entirely a part of the structure of the cerebrum. 7. The data obtained do not lend further support to the concept of axoplasmic flow within the cerebrum or the concept of a one-directional flow of mitochondria or other subcellular particles within the cerebrum.

It has been shown that [U-^{14}C]glucose injected into the intact rat is not only utilized for oxidation but a considerable portion of the glucose carbon is assimilated into proteins and lipids. Within 1hr. after injection of [^{14}C]glucose into the intact rat more than 10% of the ^{14}C could be recovered in the form of protein and lipid from the whole body of the rat (Vrba, 1966).

The process of assimilation of glucose carbon atoms into proteins *in vivo* occurs at different rates in different organs (Vrba, Gaitonde & Richter, 1962), and it would seem probable that there are differences in the rate of assimilation of glucose carbon also into various subcellular structures. This assumption has been well justified by some preliminary experiments on these lines (Vrba *et al.* 1962). The brain is unusual among other organs in its dependence on the glucose supply (Himwich, 1951), and the assimilation of glucose carbon by subcellular particles of the brain is therefore of interest. It has been previously shown that more than half of the ^{14}C of [U-^{14}C]glucose retained by the brain is incorporated within less than 15min. into brain acid-soluble amino acids (mainly dicarboxylic amino acids) (Vrba, 1962) and that within 2–5hr. the brain proteins and lipids become considerably radioactive. The first object of this work was to study the difference in the rate of incorporation of glucose carbon into proteins and lipids in various particulate matter isolated from brain homogenates. The second object was to use the process of incorporation of glucose carbon atoms into proteins and lipids of various subcellular fractions of the brain for a reinvestigation of the problem of axoplasmic flow, by using sucrose gradients to increase the degree of separation of miscellaneous

* Present address: Department of Pharmacology, University of British Columbia, Vancouver 8, B.C., Canada.

subcellular particles or particulate matter released or produced by homogenization.

The introduction of sucrose gradients by Whittaker (1961) and De Robertis (1961) has made possible a finer separation of the particulate components of brain homogenates than was available previously. A review of the potentialities and limitations of these methods has been published (Whittaker, 1965). Methods similar to those of Gray & Whittaker (1962) and of Whittaker, Michaelson & Kirkland (1964) have been applied by Barondes (1964, 1966) for an investigation of the problem of axoplasmic flow, the existence of which has been the object of numerous studies during the past two decades. These studies have been reviewed by Weiss (1961) and Ochs (1966).

In contrast with the work of Barondes (1964, 1966), who used L-[1-^{14}C]leucine, I used D-[U-^{14}C]-glucose as a precursor; this may be considered a more natural protein precursor in the brain (Vrba *et al.* 1962) since the entrance of glucose carbon into the brain is less hindered by the 'blood–brain barrier' (Dobbing, 1961). An additional advantage of using [U-^{14}C]glucose instead of L-[1-^{14}C]leucine is that glucose carbon is rapidly assimilated by brain lipids as well as by proteins (Vrba, 1962), and labelling of lipids has been in the past extensively used as a tool for the investigation of axoplasmic flow (Miani, 1963). The use of [U-^{14}C]glucose permits the problem of axoplasmic flow in brain to be studied by using as criteria both the protein and lipid constituents of the brain.

Barondes (1964, 1966) used discontinuous sucrose gradients for the separation of a fraction of the brain homogenate enriched with 'nerve-ending particles' in an attempt to demonstrate axoplasmic flow from the perikaryon towards the 'nerve-ending particles'. Discontinuous gradients were not used in the work presented below, because various fractions isolated by this method are not morphologically homogeneous and can be considered only statistically enriched with a particular morphological entity (Whittaker, 1963). Whittaker (1965) pointed out that, unless a fraction is completely homogeneous, it is usually impossible to be certain whether a particular activity is to be associated with a major component or with a minor component of high activity.

No satisfactory method is available for the isolation of all components of the brain homogenate in a morphologically homogeneous state. The crude mitochondrial fraction of rat brain was therefore subfractionated on continuous sucrose gradients and the incorporation of glucose carbon into proteins and lipids was investigated along the whole gradient, which was subdivided into 20 fractions. Each of these fractions differs by the average density and shape of its particles. The fractions obtained were identified only by consecutive numbers and I abstained in this work from using morphological names for the particles contained in the fractions, i.e. 'synoptosomes', 'nerve endings' or 'nerve-ending particles', or letters *A*, *B*, *C*, *D*, *O* etc., which are often used in the literature. My object was to find out whether it is possible by this method to isolate any particular particulate subfraction of the brain homogenate that shows a significantly higher enrichment (in comparison with other fractions or subfractions) with labelled proteins or lipids 4 days after injection of [U-^{14}C]glucose into the rat.

METHODS

Animals

Twelve male rats (40–45g. each) were used in these experiments. These were animals of the strain T_2, which is a conventionally maintained colony founded in 1965 from caesarian-derived Porton stock, and maintained by the Animals Laboratory Centre of the Medical Research Council, Carshalton, Surrey. All animals were fed *ad libitum* on M.R.C. diet (41B) until decapitation.

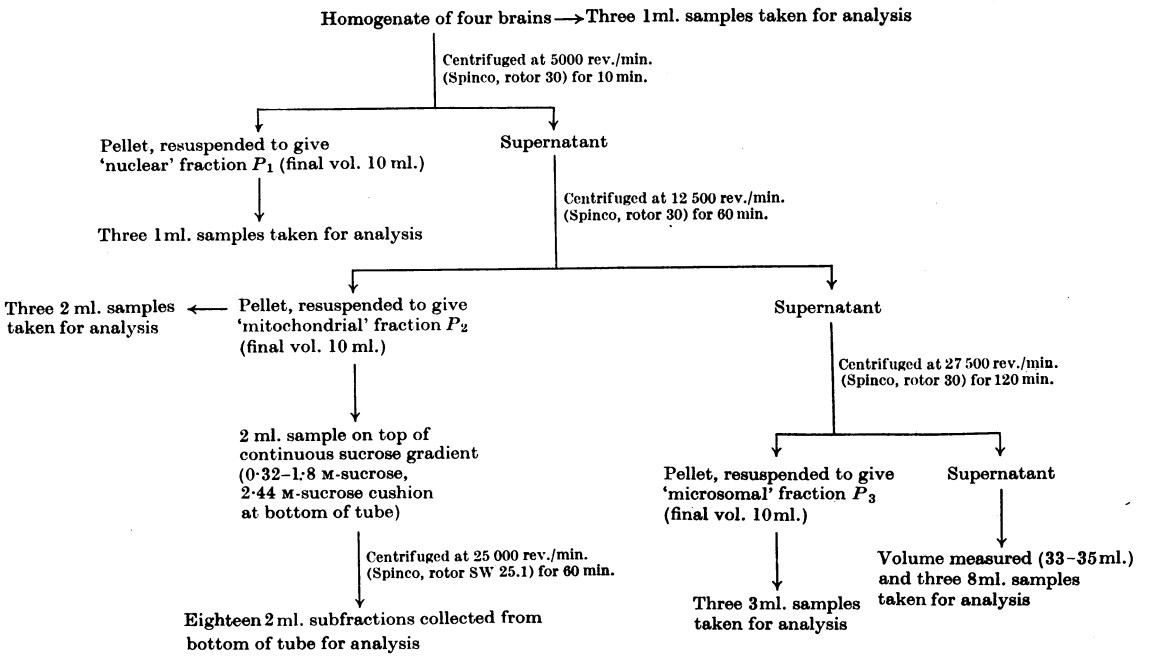
Injection of animals with [^{14}C]*glucose*

The animals were randomly divided into three groups of four animals. Group I was injected with [^{14}C]glucose 7hr. before decapitation with a guillotine. The other two groups were injected with [^{14}C]glucose at 22hr. (group II) and 93hr. (group III) before decapitation. Each animal received a subcutaneous injection of [U-^{14}C]glucose over the left flank ($81{\cdot}5 \times 10^6$ disintegrations/min.; dissolved in 0·7ml. of 0·9% NaCl in the presence of 2·17mg. of carrier glucose). The [U-^{14}C]glucose was supplied by The Radiochemical Centre, Amersham, Bucks. (batch 133).

Preparation of fractions for analysis

Initial brain dispersions. After the decapitation of the animals the brain was excised and the cerebellum removed and discarded. Four brains of each group were pooled and weighed quickly to the nearest 0·01g. They were then homogenized mechanically at 500rev./min. with a pestle clearance of 0·01in. for exactly 5min. in a homogenizer of Aldridge, Emery & Street (1960). During the homogenization in 20ml. of 0·32M-sucrose the homogenizer was immersed in a beaker filled with precooled water and ice to keep the temperature during homogenization as near as possible to 0°. (This temperature of the dispersions was maintained until the deproteinization of various fractions of the dispersion with $HClO_4$; see below.) After the homogenization the homogenizer was rinsed twice with 8ml. of 0·32M-sucrose, the washings were added to the dispersion and the whole was made up to a final volume of 40ml. with 0·32M-sucrose.

Separation of primary subcellular fractions. The no. 30 rotor of the Spinco model L ultracentrifuge was used without braking and subcellular fractions were prepared from 34ml. of each dispersion by differential centrifuging as shown in Scheme 1. The pellets obtained after each run were resuspended in 0·32M-sucrose to final volumes of 10ml.



Scheme 1. Summary of fractional centrifugation of brain dispersions. Where indicated, samples were taken for determination of protein content and measurement of ^{14}C in protein and in lipid.

Triplicate samples of these initial brain dispersions and the final supernatants were deproteinized by the addition of 0·1 vol. of 60% (w/v) $HClO_4$.

Subfractionation of the crude 'mitochondrial' fraction. Continuous sucrose gradients (25ml.) were layered over cushions of 2·44M-sucrose (4ml.) in cellulose nitrate tubes. The sucrose concentrations ranged from 1·8M above the cushions to 0·32M at the tops of the tubes. Three extra tubes were prepared. The sucrose was forced by hydrostatic pressure through syringe needles fitted through the bottoms of the tubes; 2ml. fractions were collected and the sucrose concentrations were estimated refractometrically. The average shape of these three test gradients was taken to represent those of the gradients used for the density-gradient subfractionations, which were performed at least 24hr. after their preparation. The stability and reproducibility of the sucrose gradients was constant within limits of $\pm 2\%$.

A sample (2ml.) of the crude 'mitochondrial' fraction of each experiment was layered on top of the sucrose gradients and centrifuged in the SW 25.1 rotor of the Spinco model L centrifuge for 60min. at 25000rev./min. The bottom of each cellulose nitrate tube was then punctured as described above and 2ml. fractions were collected in calibrated conical centrifuge tubes fitted with glass stoppers. The samples were immediately deproteinized by addition of 0·2ml. of 60% (w/v) $HClO_4$. The appearance of the gradient after centrifuging is shown in Fig. 1(*a*); the greater heterogeneity of the brain 'mitochondrial' fraction as compared with that from liver is shown by the inclusion of a parallel experiment with the latter tissue (Fig. 1*b*) (cf. van Kempen, van den Berg, van der Helm & Veldstra, 1965).

Protein purification and estimation, and extraction of lipids

The procedure is summarized in Scheme 2. The volumes of the deproteinized samples were made up to 10ml. with 6% (w/v) $HClO_4$, rotated for 1hr. in a Matburn blood-cell-suspension mixer (Matburn Ltd., London, W.C. 1) and then centrifuged at 0° in an MSE Major centrifuge at 1500rev./min. The supernatant was discarded. The residue was resuspended in 10ml. of 6% (w/v) $HClO_4$ in a Whirlimixer (Fisons Scientific Apparatus Ltd., Loughborough, Leics.), centrifuged and the supernatant discarded. This procedure of cold extraction with 6% (w/v) $HClO_4$ was repeated four times to ensure elimination of acid-soluble ^{14}C-labelled compounds.

The acid-insoluble sediment was resuspended in 10ml. of ethanol–acetone (1:1, v/v) and extracted by rotation in the blood-cell mixer for 1hr., then centrifuged in the MSE Major centrifuge for 30min. at 1500rev./min. The supernatant was collected in 25ml. graduated glass-stoppered test tubes. The sediment was resuspended as described above in 10ml. of chloroform–methanol–diethyl ether (2:1:1, by vol.), extracted for 1hr. by rotation in the blood-cell mixer and centrifuged as above for 30min. at 1500rev./min. The same procedure was repeated with

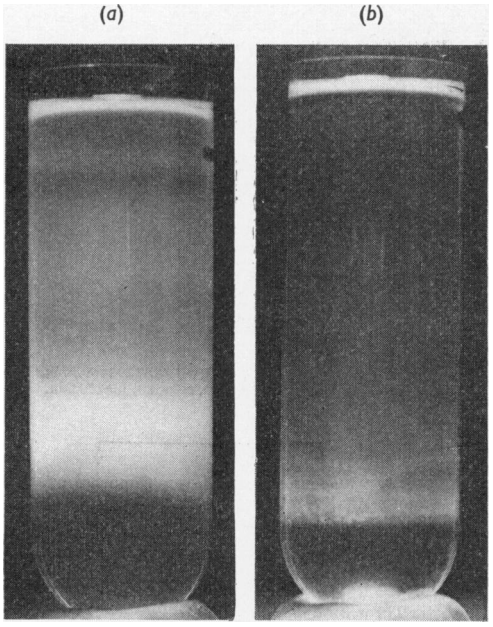
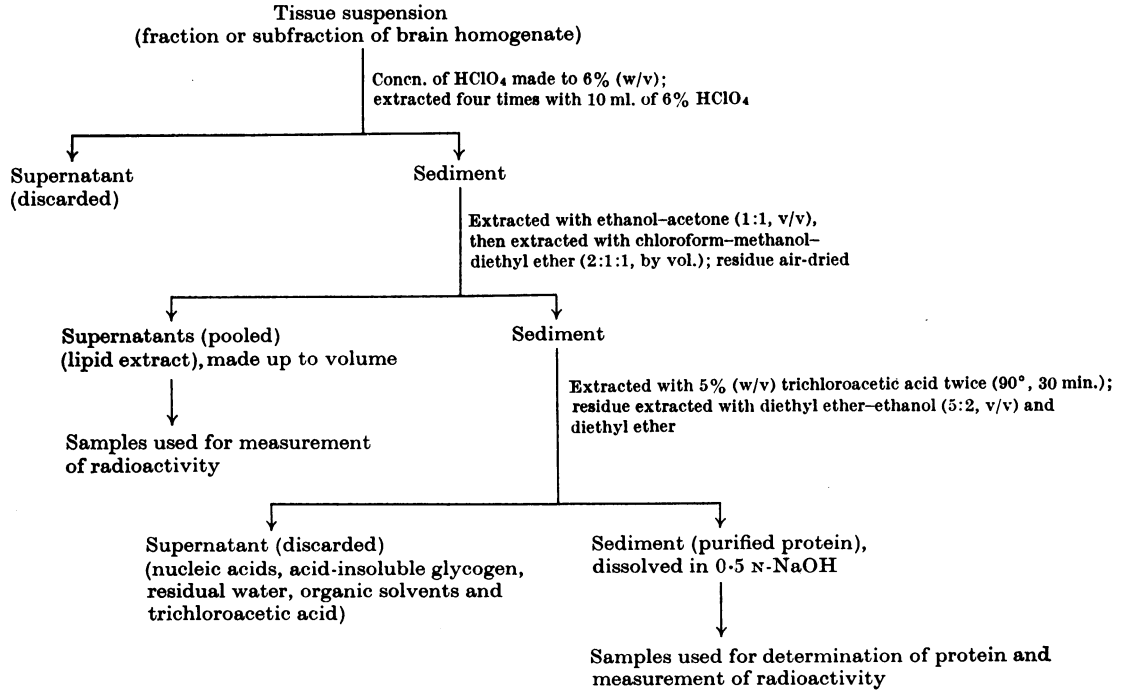


Fig. 1. Subfractionation of crude brain (*a*) and liver (*b*) 'mitochondrial' P_2 fractions on continuous sucrose gradients. Rat brain (four brains, 3·87g.) or liver (1·74g.) tissue was homogenized, and subcellular fractions were prepared and subfractionated by using sucrose density gradients as described in the Methods section.

5ml. of Bloor's mixture (ethanol–diethyl ether; 3:1, v/v). The combined extracts were made up to 25ml. with diethyl ether and kept for the estimation of radioactivity.

The residue after lipid extraction was dried at 80° in a water bath, resuspended in 3ml. of 5% (w/v) trichloroacetic acid and incubated at 90° in a water bath with constant shaking. On cooling, the contents were centrifuged in an MSE Major centrifuge at 2000rev./min. and the supernatant was removed by suction. The hot extraction with trichloroacetic acid was repeated twice, and the residue was suspended in 8ml. of ethanol–diethyl ether (2:5, v/v) and extracted for 1hr. by rotation in the blood-cell mixer. After centrifugation at 1500rev./min. for 30min. the supernatant was sucked off and discarded, the residue resuspended in 8ml. of diethyl ether and the procedure of extraction, centrifugation and removal of supernatant repeated. The procedure was designed to remove acid-insoluble glycogen, nucleic acids and adsorbed radioactive acid-soluble compounds, and finally to remove traces of $HClO_4$ and trichloroacetic acid (Vrba, 1966). The residue ('purified protein') was air-dried and dissolved in 10ml. of 0·5N-NaOH in all cases except for the subfractions of fraction P_2, which were dissolved in 2ml. of 0·5N-NaOH.

Protein in these solutions was estimated by a minor modification of the method of Lowry, Rosebrough, Farr & Randall (1951); 25–200μl. samples (containing 30–100μg. of protein) were diluted (if necessary) to a final volume of 200μl. with 0·5N-NaOH, and 0·8ml. of water was added. Reagent C of Lowry *et al.* (1951) (5ml.) was added, mixed with a Whirlimixer and allowed to stand for 10min. Their reagent E (0·5ml.) was then added, the whole mixed immediately and after 30min. the colour read at 500mμ



Scheme 2. Summary of the procedure for purification of proteins and preparation of lipid extracts.

in a Unicam SP.600 spectrophotometer (Unicam Instruments, Cambridge). Under these conditions 100μg. of bovine albumin in a final volume of 6·5ml. gave $E^{1\,cm.}_{500\,m\mu}$ 0·178. Crystallized bovine serum albumin (20–200μg.) (Sigma Chemical Co., St Louis 18, Mo., U.S.A.) served as a standard. All samples and standards were analysed in triplicate; the standards were carried through the analytical procedure together with the samples.

Measurements of radioactivity

Proteins. The radioactivity of the NaOH solutions was counted in a Nuclear–Chicago (ambient-temperature model) liquid-scintillation counter. Each solution was measured with two different sample volumes (0·2–0·8ml.) depending on its radioactivity. The samples were dissolved in 12ml. of scintillator of the following composition (Hall & Cocking, 1965): 0·4% (w/v) 2,5-diphenyloxazole and 0·01% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene–2-ethoxyethanol (7:3, v/v). Then 0·1ml. of conc. formic acid was added to each sample. This scintillator alone was not sufficient to keep the samples in solution, and therefore 5ml. of ethanol containing 4% (v/v) of conc. formic acid was added to each vial. Under these conditions the radioactivity increased linearly with the amount of added sample. Counts/min. were converted into distintegrations/min. with the help of a reproducible quenching curve, prepared from a standard *n*-[1-^{14}C]hexadecane solution in the same scintillator mixture and quenched by the addition of 0·1–0·5ml. of N-NaOH or 0·1–0·9ml. of $CHCl_3$. For calculation of results the two-channel-ratio method was used. For further control each sample was counted again in the presence of an internal reference standard of *n*-[1-^{14}C]hexadecane (0·781μc/g.; supplied by The Radiochemical Centre). The average recovery of added reference standard, after conversion of counts/min. into disintegrations/min., was 99%. The standard deviation, calculated from 50 randomly chosen experiments, was 10%. Each sample was counted for 20min. (2000–50000counts according to the radioactivity) and the absolute efficiency varied in the range 45–75%. As described above, all results were calculated to 100% efficiency.

Lipids. The lipid extracts contained traces of $HClO_4$, as the lipids had been extracted after $HClO_4$ treatment. Therefore samples of the lipid extract were neutralized before evaporation to prevent charring due to gradual concentration of $HClO_4$ during solvent evaporation. Samples (5ml.) of each lipid extract were pipetted into scintillation vials, 1 drop of phenolphthalein [1% (w/v) solution in 50% (v/v) ethanol] was added and then each sample was neutralized by the addition of 30% (w/v) KOH until a permanent red colour appeared. The excess of KOH was eliminated by the addition of N-HCl until decolorization of the phenolphthalein occurred and an additional 5–6 drops of N-HCl was added. The organic solvents and the surplus of HCl were then evaporated in a desiccator *in vacuo* over NaOH pellets to eliminate the last traces of HCl. The residue was finally dried under an infrared lamp in a fume cupboard, and dissolved in 10ml. of a toluene scintillator [0·03% (w/v) 2,5-diphenyloxazole and 0·5% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene]. Each lipid sample was measured by the two-channel-ratio method (with a quenching curve) and also with the help of an internal standard. Both methods gave practically identical results, the recovery of added reference standard of *n*-[1-^{14}C]hexadecane, after conversion of counts/min. into disintegrations/min., averaged 97·5%. The S.D., calculated from 20 randomly chosen measurements, was 9·5%. From the calculated S.D. I conclude that differences of more than 20% (2 × S.D.) may be considered as the lower limit of a significant difference.

RESULTS

Proteins. The recovery of protein from the four main crude fractions was about 90% (Table 1, section A), which is satisfactory for this study as the S.D. of the ^{14}C determinations was 10%. The distribution of the protein in the four crude fractions was reproducible (Table 1, section B); in each case 53–54% of the brain protein appeared in the P_2 fraction. This constant protein content was in sharp contrast with the change in the distribution of radioactivity among the proteins of the four crude subcellular fractions 93hr. after the injection of [U-^{14}C]glucose (Table 1, section D). The increase of ^{14}C in the P_2 fraction between 22 and 93hr. after the injection of [U-^{14}C]glucose was accompanied by a commensurate decrease of the percentage of radioactivity in the protein of the supernatant fraction (Table 1, section D). These results would appear to confirm the findings of Barondes (1964) of a 'specific' shift of labelled proteins from the supernatant fraction into the 'mitochondrial' fraction (which would be expected to contain the 'nerve-ending particles') and thus lend support to the concept of 'axoplasmic flow' inside the cerebrum.

The total amount of ^{14}C in the brain proteins of the original homogenate was unchanged during the period 7–22hr. after the injection of [U-^{14}C]glucose, but thereafter during the period 22–93hr. the amount of ^{14}C in the proteins of the brain homogenate was markedly decreased (Table 1, section C). This can be explained as a consequence of the constant turnover of brain proteins, but different relative turnover rates in different fractions may be assumed. The specific activity of the proteins of all except the P_2 fraction was decreased. The specific activity of proteins of the P_2 fraction increased in the period 22–93hr. after the injection of [U-^{14}C]-glucose, whereas the specific activities of proteins of all other fractions decreased considerably (Table 1, section E). This is in good agreement with similar experiments of Barondes (1964). The picture was similar if the results are expressed in terms of relative specific activity ($R.S.A._n$), where average specific activity of the protein of the whole brain homogenate is taken to be 100 in each case. The results expressed in this way take into account the constant loss of ^{14}C from proteins of the brain in general (Table 1, section F). Even these data

Table 1. *Distribution of labelled protein in subcellular fractions from rat brain*

For the experimental design and nomenclature of fractions see the text. Each value is the result of the analysis of four pooled brains.

		Expt. I	Expt. II	Expt. III
Time after injection of [U-^{14}C]glucose		7 hr.	22 hr.	93 hr.
Fresh wt. of four brains		4·08 g.	4·22 g.	3·91 g.
Total ^{14}C recovered from cerebral proteins (% of injected dose)		0·071	0·078	0·057
A Amount of protein (mg.)	Original homogenate	291	308	286
	Fraction P_1	53	58	50
	Fraction P_2	138	148	137
	Fraction P_3	11	12	11
	Supernatant	58	61	57
	Recovery of protein (%)	89	91	89
B Distribution of protein (% of total recovered)	Fraction P_1	20·4	20·8	19·6
	Fraction P_2	53·1	53·0	53·7
	Fraction P_3	4·2	4·3	4·3
	Supernatant	22·3	21·9	22·4
C $10^{-3}\times$ Amount of ^{14}C in protein (disintegrations/min.)	Original homogenate	231	254	186
	Fraction P_1	38	46	32
	Fraction P_2	77	86	89
	Fraction P_3	11	12	9
	Supernatant	60	64	39
	Recovery of ^{14}C (%)	81	82	90
D Distribution of ^{14}C in protein (% of total recovered in protein)	Fraction P_1	20·4	22·1	18·9
	Fraction P_2	41·4	41·3	52·7*
	Fraction P_3	5·9	5·8	5·3
	Supernatant	32·3	30·8	23·1*
E Sp. activity of protein (disintegrations/min./mg. of protein)	Original homogenate	794	825	650*
	Fraction P_1	717	793	640
	Fraction P_2	558	581	650
	Fraction P_3	1000	1000	818
	Supernatant	1034	1049	684*
F Relative sp. activity of protein ($R.S.A._h$) (sp. activity of proteins of original homogenate = 100)	Original homogenate	100	100	100
	Fraction P_1	90	96	98
	Fraction P_2	70	70	100*
	Fraction P_3	126	121	126
	Supernatant	130	127	105*
G Relative sp. activity of protein ($R.S.A._s$) (sp. activity of proteins of high-speed supernatant = 100)	Original homogenate	77	79	105*
	Fraction P_1	69	76	93*
	Fraction P_2	54	55	95*
	Fraction P_3	97	95	119*
	Supernatant	100	100	100

* Statistically significant difference with time:

$$\Delta > 2 \text{ S.D.}; \text{ S.D.} = \sqrt{\frac{\Sigma\Delta^2}{n-1}} = 10\%.$$

might not be considered to conflict with those of Barondes (1964), who assumed that labelled proteins 'flow' through the axoplasm specifically into the 'nerve-ending particles', which are assumed to enrich the P_2 fraction (Barondes, 1964).

Quite a different picture emerges if it is assumed that there is no specific flow of proteins of the supernatant fraction into the 'mitochondrial' (P_2) fraction, but that some soluble proteins, synthesized from glucose carbon in the soluble cytoplasm outside the organelles, penetrate to a certain degree into most of the subcellular particulate matter. It is evident from Table 1 (section E) that the supernatant proteins show a higher specific activity than any other particulate proteins during the first 22 hr. after the injection of [U-^{14}C]glucose. To test the assumption of a diffusion of at least some of the supernatant proteins into various types of particulate fractions, the specific activity of the soluble supernatant protein has to be taken as 100,

Table 2. *Distribution of radioactivity in the proteins of subfractionations of the* P_2 *fraction, prepared by sucrose-density-gradient centrifugation*

The results represent the analysis of pooled 2ml. fractions; the pooling into three components ('heavy': nos. 6–12; 'medium': nos. 13–16; 'light': nos. 17–19) was performed on the basis of the shape of the curve of protein distribution along the gradient, as recorded in Fig. 2. For nomenclature of fractions and experimental details see the text. Each value is the result of the analysis of four pooled rat brains.

			Expt. I	Expt. II	Expt. III
	Time after injection of [U-^{14}C]glucose		7hr.	22hr.	93hr.
A	Amount of protein (mg.)	Fraction P_2	138	148	137
		'Heavy' subfraction	92	125	112
		'Medium' subfraction	24	21	19
		'Light' subfraction	12	10	16
B	$10^{-3}\times$ Amount of ^{14}C in protein (disintegrations/min.)	Fraction P_2	77	86	89
		'Heavy' subfraction	47	75	76*
		'Medium' subfraction	14	18	14
		'Light' subfraction	10	10	13
C	Sp. activity of protein (disintegrations/min./mg. of protein)	Fraction P_2	558	581	650
		'Heavy' subfraction	511	600	679*
		'Medium' subfraction	583	857	737*
		'Light' subfraction	833	1000	812
D	Relative sp. activity of protein ($R.S.A._h$) (sp. activity of proteins of original homogenate = 100)	Fraction P_2	70	70	100*
		'Heavy' subfraction	64	73	104*
		'Medium' subfraction	73	104	113*
		'Light' subfraction	105	121	125
E	Relative sp. activity of protein ($R.S.A._s$) (sp. activity of proteins of high-speed supernatant = 100)	Fraction P_2	54	55	95*
		'Heavy' subfraction	49	57	99*
		'Medium' subfraction	56	82	108*
		'Light' subfraction	102	115	119

* Statistically significant difference with time (see footnote to Table 1).

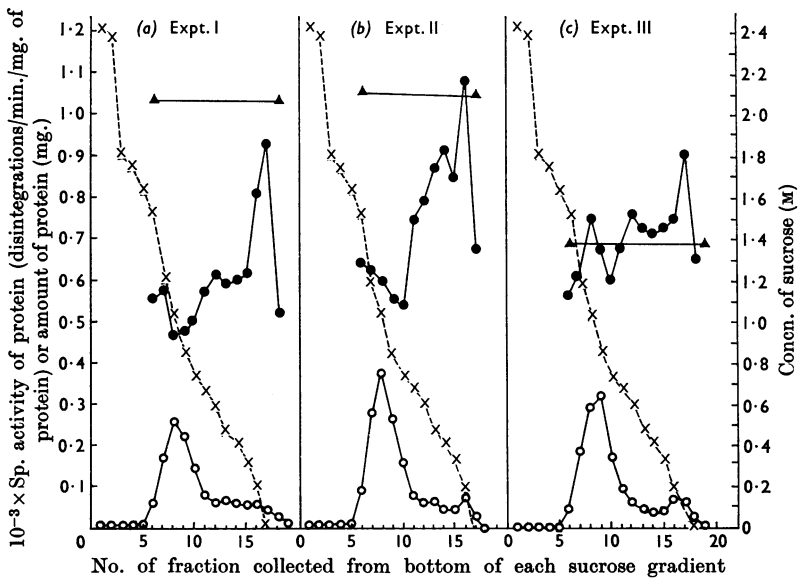


Fig. 2. Subfractionation of the crude brain 'mitochondrial' P_2 fractions on continuous sucrose gradients: protein analysis. Experimental conditions are described in the Methods section. ●, Specific activity of protein; ○, amount of protein in each subfraction; ×, concn. of sucrose along the gradient; ▲, specific activity of the protein of the high-speed brain supernatant of the same experiment. (*a*) Expt. I, 7hr. after injection of [U-^{14}C]glucose; (*b*) Expt. II, 22hr. after injection of [U-^{14}C]glucose; (*c*) Expt. III, 93hr. after injection of [U-^{14}C]glucose.

Table 3. *Subcellular distribution of radioactivity in rat brain lipids after injection with* [U-^{14}C]*glucose*

For details see the Methods section.

		Expt. II	Expt. III
Time after injection of [U-^{14}C]glucose		22hr.	93hr.
Total ^{14}C recovered from cerebral lipids (% of injected dose)		0·048	0·048
$10^{-3}\times$ Amount of ^{14}C in lipid (disintegrations/min.)	Original homogenate	156	156
	Fraction P_1	35	38
	Fraction P_2	110	108
	Fraction P_3	8	9
	Supernatant	4	3
	Recovery of ^{14}C (%)	100·6	101·3
Distribution of ^{14}C in lipid (% of total recovered in lipid)	Fraction P_1	22·3	24·0
	Fraction P_2	70·0	68·3
	Fraction P_3	5·1	5·7
	Supernatant	2·5	1·9

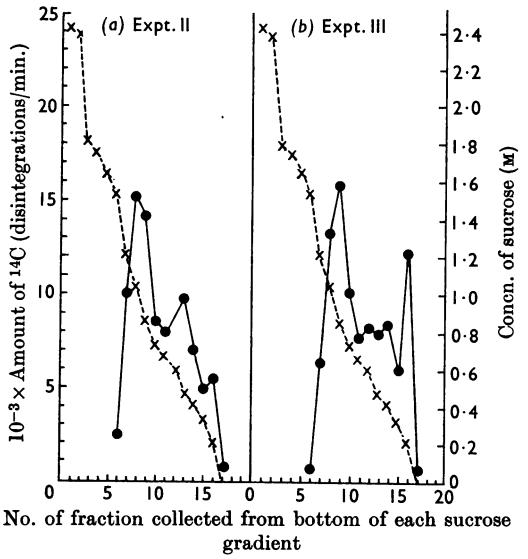


Fig. 3. Radioactivity of lipids extracted from subfractions of the crude 'mitochondrial' P_2 fraction prepared as described in the Methods section. ●, Amount of ^{14}C in the lipid of each subfraction; ×, concn. of sucrose along the gradient. (*a*) Expt. II, 22hr. after injection of [U-^{14}C]-glucose; (*b*) Expt. III, 93hr. after injection of [U-^{14}C]-glucose.

and the relative specific activities of all other insoluble (i.e. particulate) fractions expressed on this basis (R.S.A.$_s$). The results obtained by these calculations are recorded in Table 1 (section G) and fully confirm my assumption. The relative specific activities (R.S.A.$_s$) of all insoluble particles of the brain homogenate were constantly increasing during the whole experimental period 7–93hr. after the injection of [U-^{14}C]glucose. This general increase of R.S.A.$_s$ is statistically significant.

The results in Table 2 indicate that this conclusion is valid not only for crude particulate fractions (P_1, P_2 and P_3), but also for the subfractions of the P_2 fraction. In both 'heavy' and 'medium' subfractions of the P_2 fraction there is a steady and significant increase of R.S.A.$_h$ and R.S.A.$_s$ in the period 22–93hr. after injection of [U-^{14}C]glucose, but this increase is larger in the 'heavy' subfraction than in the 'medium' subfraction of fraction P_2 (Table 2, sections C, D and E). This is in marked contrast with the constant amount of protein found in these fractions (Table 2, section A).

Fig. 2 shows that the specific activity of the protein of all subfractions of the crude 'mitochondrial' fraction tended with time to approach the specific activity of the protein of the high-speed soluble supernatant. The change in radioactivity with time was not confined to any one subfraction. Fig. 2 and Table 1 indicate that considerable diffusion of soluble proteins into all particulate fractions and subfractions of the cerebrum takes place. No evidence was obtained that would justify the assumption that there is an orientated one-directional 'flow' of soluble proteins into any particular subfraction of the crude 'mitochondrial' fraction.

Lipids. The above conclusion is fully supported by the analysis of radioactivity in the brain lipids, both in the crude subcellular fractions (Table 3) and in the 'mitochondrial' subfractions (Fig. 3). Table 3 shows that, in spite of the complete recovery of the radioactivity, no changes in the distribution of ^{14}C among lipids of the four crude subcellular fractions (P_1, P_2, P_3 and S) of the cerebrum were observed between 22 and 93hr. after the injection of [U-^{14}C]glucose. Analysis of ^{14}C in the lipids of the 'mitochondrial' subfractions lends further support to this conclusion (Fig. 3).

Comparison of the data of Tables 1 and 3 suggests two basic differences between the proteins and

lipids of brain, as far as the assimilation of glucose carbon is concerned.

First, the absolute amounts of glucose carbon incorporated by brain proteins or by brain lipids were similar, but the percentage distributions of the incorporation into lipids and proteins of different subcellular fractions were different. About 23–33% of the glucose carbon assimilated by brain proteins was recovered in the high-speed supernatant fraction (Table 1, section D), whereas less than 3% of the glucose carbon assimilated by brain lipids was found in this subcellular fraction (Table 3). On the other hand, the lipids of the 'mitochondrial' fraction assimilated glucose carbon even more readily than did the proteins of that fraction. There was virtually no difference in the rates of ^{14}C incorporation by proteins and lipids between the crude P_1 and P_3 fractions. It is noteworthy that 5hr. after the injection of [U-^{14}C]glucose the specific activity of the protein in the 'myelinated' structures of the P_1 fraction is not very different from that of the protein of the 'nuclei' (Vrba *et al.* 1962). The 'nuclei' in those experiments were separated from the 'myelinated' structures by the method of Borkowski, Harth, Mardell & Mandell (1961).

Secondly, a shift of the labelled proteins from the high-speed supernatant into particulate subfractions during 93hr. after injection of [U-^{14}C]glucose was observed (Table 1, sections D, F and G), whereas no such shift of lipids occurred (Table 3). These observations are in full accordance with the fact that a part of the brain proteins is in solution and therefore diffusible, whereas the brain lipids occur in the cerebrum almost entirely in a non-diffusible state, as they constitute a part of the structure of the cerebrum.

DISCUSSION

The retention and assimilation of [U-^{14}C]glucose carbon is higher in brain than in liver, kidney, heart, spleen, muscle, lung or blood (Vrba *et al.* 1962). It is not valid to conclude from these data that these processes are more active in the brain than in all other organs (Gaitonde, Richter & Vrba, 1962). Steele (1954) studied the retention of [U-^{14}C]-sucrose after ingestion by male C_{57} black mice He found that in the bone marrow this process was almost as intensive as in the brain, whereas colon and rectum, ileum and jejunum, large intestine, cervical lymph node, salivary gland, stomach and thymus were superior to the brain with respect to sucrose carbon assimilation. For purposes of rough approximation we may assume that the brain represents only an average between all body organs in its ability to retain and assimilate [^{14}C]glucose carbon, if we also accept, using Steele's (1954) data, that the fate of sucrose carbon *in vivo* is not principally dissimilar to that of glucose carbon, which might be expected since sucrose will be converted into glucose before it is utilized.

About 10% of the administered [^{14}C]glucose carbon is converted into proteins and lipids in the body of the mouse (Vrba, 1966). The average weight of the rats used in the present experiments was about 42g. and the average fresh weight of the brain was about 1g.; this represents 2·4% of the average body weight. The amount of glucose carbon assimilated by the brain proteins and lipids should be expected (with all the above approximations) to be 10% of 2·4%, i.e. 0·24% of the injected dose of [^{14}C]glucose. Tables 1 and 3 show that the brain proteins assimilated 0·071% of the injected [^{14}C]glucose carbon and the brain lipids assimilated 0·047%; this represents together 0·12% of the injected glucose carbon. This is in satisfactory agreement with the value of 0·24% deduced from data obtained from different experiments by different authors who made use of either rats or mice of various strains (Steele, 1954; Vrba *et al.* 1962; Vrba, 1966).

No convincing evidence for the existence of a directed axonal flow in the cerebrum could be demonstrated. However, evidence was obtained in the present study for the diffusion of some brain supernatant protein into almost all particulate matter of the brain homogenate (Fig. 2). Diffusion of certain soluble proteins of the cell sap into mitochondria in other biological systems was described by Haldar, Freeman & Work (1966). Synthesis of proteins in isolated rat brain mitochondria has also been established (Bachelard, 1966). The findings of Haldar *et al.* (1966) and of Bachelard (1966) may be relevant to my observations.

The results presented above show that there is no evidence for the existence of axoplasmic flow within the cerebrum from conclusions based on protein analysis. The high radioactivity of the lipids of the brain mitochondria, the 100% recovery of ^{14}C from all subcellular fractions of the rat brain dispersion and the constancy of the subcellular distribution of ^{14}C among the lipids of the subcellular particles (Table 3) virtually excludes any orientated one-directional movement of mitochondria or other subcellular particles within the cerebrum.

The results of Barondes (1964) could lead to an identical conclusion if based on a calculation of relative specific activities rather than specific activities. Recalculation of the results in Table 2 of his paper shows that the relative specific activity of the proteins of all particulate matter in his experiments increased with time (R.S.A. = specific activity of proteins of a particulate fraction/specific

activity of proteins of the whole homogenate). The special position of the 'nerve endings' disappears and with it the evidence for axoplasmic flow.

I am grateful to Miss Angela Sheehan for her careful and conscientious technical assistance.

REFERENCES

Aldridge, W. N., Emery, R. C. & Street, B. W. (1960). *Biochem. J.* **77**, 326.
Bachelard, H. S. (1966). *Biochem. J.* **100**, 131.
Barondes, S. H. (1964). *Science*, **146**, 779.
Barondes, S. H. (1966). *J. Neurochem.* **13**, 721.
Borkowski, T., Harth, S., Mardell, R. & Mandel, P. (1961). *Nature, Lond.*, **192**, 456.
De Robertis, E. (1961). *Proc. 4th int. Neurochem. Symp.: Regional Neurochemistry, Varenna*, p. 248. Ed. by Kety, S. S. & Elkes, J. Oxford: Pergamon Press Ltd.
Dobbing, J. (1961). *Physiol. Rev.* **41**, 130.
Gaitonde, M. K., Richter, D. & Vrba, R. (1962). *Biochem. J.* **84**, 105P.
Gray, E. G. & Whittaker, V. P. (1962). *J. Anat., Lond.*, **96**, 79.
Haldar, D., Freeman, K. & Work, T. S. (1966). *Nature, Lond.*, **211**, 9.
Hall, T. C. & Cocking, E. C. (1965). *Biochem. J.* **96**, 626.
Himwich, H. E. (1951). *Brain Metabolism and Cerebral Disorders*, pp. 24–25. Baltimore: The Williams and Wilkins Co.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Miani, N. (1963). *J. Neurochem.* **10**, 859.
Ochs, S. (1966). In *Macromolecules and Behaviour*, p. 20. Ed. by Gaito, J. Amsterdam: North-Holland Publishing Co.
Steele, R. (1954). *J. biol. Chem.* **209**, 91.
van Kempen, G. M. J., van den Berg, C. J., van der Helm, H. J. & Veldstra, H. (1965). *J. Neurochem.* **12**, 581.
Vrba, R. (1962). *Nature, Lond.*, **195**, 663.
Vrba, R. (1966). *Biochem. J.* **99**, 367.
Vrba, R., Gaitonde, M. K. & Richter, D. (1962). *J. Neurochem.* **9**, 465.
Weiss, P. (1961). *Proc. 4th int. Neurochem. Symp.: Regional Neurochemistry, Varenna*, p. 220. Ed. by Kety, S. S. & Elkes, J. Oxford: Pergamon Press Ltd.
Whittaker, V. P. (1961). *Proc. 4th int. Neurochem. Symp.: Regional Neurochemistry, Varenna*, p. 259. Ed. by Kety, S. S. & Elkes, J. Oxford: Pergamon Press Ltd.
Whittaker, V. P. (1963). *Symp. biochem. Soc.* **23**, 109.
Whittaker, V. P. (1965). *Progr. Biophys. mclec. Biol.* **15**, 39.
Whittaker, V. P., Michaelson, J. A. & Kirkland, R. J. A. (1964). *Biochem. J.* **90**, 293.